

vanced coronary plaque vulnerability.

In case 1, coronary intervention and antiplatelet medication without glycoprotein IIb-IIIa inhibitors was not sufficient to treat severe coronary plaque instability and platelet hyperactivity, as indicated by excessive and persistent whole-blood hypercholinemia ( $>100 \mu\text{mol/L}$ ), and the patient died of sudden cardiac death shortly after hospital discharge.

In case 2, WBCHO was the only test indicating evolving stent occlusion at an early stage, whereas other biomarkers such as cTnI, myoglobin, D-dimers, and the ECG were nondiagnostic. We were lucky that case 2 returned to the emergency room the next day. The integration of choline testing into routine clinical decision-making would have prevented inappropriate discharge from the emergency room. Whole-blood hypercholinemia in the setting of evolving stent thrombosis after clopidogrel withdrawal clearly points to platelet hyperactivity as a major component for increased WBCHO in this patient.

In case 3, high concentrations of WBCHO were present in a massive thrombosis of a coronary bypass graft requiring aspiration-thrombectomy and stent implantation. This case is interesting because it demonstrates that exceptionally high concentrations of WBCHO ( $>100 \mu\text{mol/L}$ ) may evolve in forms of massive intravascular (platelet) thrombosis, whereas other thrombosis markers (D-dimers) remain low.

In contrast to WBCHO concentrations, the PLCHO concentrations were low in all 3 cases. The difference between WBCHO and PLCHO concentrations are explained by several mechanisms: intracellular generation of choline in blood cells by intracellularly located phospholipase D and cell activation pathways (3–7), existence of a choline transport system in blood cells (10), and the fact that choline is removed to a certain extent from plasma via cellular uptake by other tissues (11). PLCHO is also a significant predictor of cardiac events, and concentrations increase in acute coronary syndromes complicated by acute left-ventricular failure and severe tissue ischemia (2), which

was not evident in these cases. PLCHO concentrations were helpful in identifying the risk associated with phospholipase D activation in these patients more precisely as related to coronary plaque destabilization and coronary platelet thrombosis rather than to tissue ischemia.

It should be emphasized that these cases were selected for a discussion on the pathophysiology of whole-blood hypercholinemia in the absence of increased PLCHO and are not suitable for generalized conclusions on the differential clinical value of these markers, which should be based on clinical trials. Individual concentrations of whole blood and plasma choline have to be interpreted with respect to the pathophysiology of acute coronary syndromes (12). Early biochemical detection of high-risk patients, as presented in these case reports, remains an important issue to target potentially life-saving advanced treatment strategies and to perform coronary angiography and intervention early and with sustained success.

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#### Circulating Nucleic Acids in Blood of Healthy Male and Female Donors

To the Editor:

The analysis of circulating nucleic acids (cirNAs) in blood provides helpful information for medical diagnostics (1). Thus, there has been intensive investigation of the factors influencing nucleic acid concentrations in plasma and serum, such as changes in DNA concentrations in serum and plasma during storage of blood samples (2) and the influence of blood processing on cell-free DNA quantification (3–5). Recent observations demonstrated that cirNAs are found not only free in blood plasma but also bound to the cell surface (6).

Physiologic factors determining cirNA concentrations in blood remain to be elucidated. In the present study, we compared the concentration of cell-surface-bound and cell-free cirNAs in blood samples from 15 healthy men and 20 healthy women.

Venous blood (8 mL) was collected into 13-mL tubes (cat. no. 55.459; Sarstedt) containing 2 mL of sterile phosphate-buffered saline (PBS) containing 50 mmol/L EDTA. The samples were stored at 4 °C and treated within 4 h after blood collection. Plasma was collected after low-speed centrifugation (400g for 20 min) followed by high-speed centrifugation (16 000g for 5 min). Blood cells were fractionated into leukocytes and erythrocytes, and extracellular nucleic acids were eluted from the cell surface with PBS containing 5 mmol/L EDTA (PBS/EDTA) and trypsin solutions as described previously (6). Briefly, cells were washed with 9 volumes of PBS/EDTA and centrifuged (400g for 10 min). Cells were resuspended and incubated for 5 min at room temperature with 2.5 g/L trypsin with subsequent inactivation of the enzyme by trypsin inhibitor. Cells were then pelleted by centrifugation, and the supernatant was collected as trypsin eluate. RNA and DNA were isolated (7) and quantified by a fluorescence-based method (8). Plasma (1 mL), PBS/EDTA eluate (2 mL), and trypsin eluate (1 mL) were used for cirNA quantification. Detection limits for RNA and DNA calculated to the initial blood volume were 8 ng/mL in plasma, 20 ng/mL in trypsin eluate, and 40 ng/mL in PBS/EDTA eluate. Statistical tests were carried out with STATISTICA 6.0 software.

The mean (SD) concentration of circulating DNA (cirDNA) in plasma of healthy men did not exceed 16 (7) ng/mL of total blood (Table 1; also see Table 1 of the Data Supplement that accompanies the online version of this letter at <http://www.clinchem.org/content/vol51/issue7/>) and was similar to the mean cirDNA concentration in plasma of healthy women [15 (13) ng/mL of total blood], in accordance with published data (9). Detectable concentrations of

**Table 1. cirNAs in the blood of healthy donors.**  
Cell-surface-bound cirNAs

Sex	Age, years	Mean, <sup>a</sup> ng/mL	Incidence, n (%)	cirNAs in plasma			Cell-surface-bound cirNAs						
				DNA	RNA	n	PBS/EDTA eluate		Trypsin eluate		Total cirNAs in blood		
							DNA	RNA	DNA	RNA	DNA	RNA	
M	18–43	16 (7) <sup>b</sup>	126 (120–140) <sup>c</sup>	298 (70–780) <sup>c</sup>	186 (40–380) <sup>c</sup>	682 (70–2030) <sup>c</sup>	422 (100–1340) <sup>c</sup>	92 (20–460) <sup>c</sup>	169 (20–810) <sup>c</sup>	246 (50–1890) <sup>c</sup>	227 (50–990) <sup>c</sup>	1030 (210–2250) <sup>c</sup>	770 (90–2280) <sup>c</sup>
F	25–53	15 (13) <sup>b</sup>	5 (33) (14–60) <sup>c</sup>	8 (53) (40–100) <sup>c</sup>	12 (80) (40–220) <sup>c</sup>	10 (67) (40–320) <sup>c</sup>	9 (60) (40–140) <sup>c</sup>	15 (100) (30–870) <sup>c</sup>	12 (80) (20–90) <sup>c</sup>	14 (95) (20–320) <sup>c</sup>	13 (87) (20–100) <sup>c</sup>	15 (100) (80–900) <sup>c</sup>	15 (100) (30–440) <sup>c</sup>

<sup>a</sup> Concentrations of cirNAs are calculated to 1 mL of blood.

<sup>b</sup> Mean (SD) for gaussian distribution.

<sup>c</sup> Mean (range) for nongaussian distribution.

circulating RNA (cirRNA) were found in blood plasma of 33% of healthy men and 35% of healthy women. Notably, the cirRNA concentration in plasma was higher in men than in women (Table 1; also see Table 1 of the online Data Supplement).

We did not find any correlation between the ages of the patients and the concentrations of free or cell-surface-bound cirNAs. The total mean concentration of cirDNA in blood was higher in healthy men (1030 ng/mL of blood) than in healthy women (430 ng/mL; Mann-Whitney *U*-test, *P* < 0.01). The cirRNA concentration was also significantly higher in men than in women (770 vs 100 ng/mL of blood; Mann-Whitney *U*-test, *P* < 0.01).

We have found that the main portion of cirNAs in healthy donors is tightly or weakly bound to the cell surface, apparently because of interaction with cell-surface NA-binding proteins (10) or phospholipids of the cellular membrane through bivalent ions (11). In the blood of breast cancer patients, cirNAs circulate only in blood plasma and are not detected at the cell surface (6). This finding indicates that the cirNA concentration in plasma is determined not only by the intensity of cell death (12) or by active secretion from cells (13) but also by the interaction of cirNAs with cell surface and plasma biopolymers. Increased concentrations of proteases accompanying tumor invasion in cancer patients (14, 15) can cause damage to the cell-surface NA-binding proteins and detachment of the cell-surface-bound cirNAs. Proteolytic enzymes are also responsible for endometrium turnover in the uterus (16), and background protease activity combined with known high nuclease activity in blood plasma (17) can affect the concentrations and distribution of cirNAs in the blood of healthy female donors and sick female patients.

The results of the present study demonstrate that although the concentration of cell-free cirDNA in the

plasma of healthy donors does not depend on the sex of the donors, concentrations of cell-surface-bound cirNA differ in male and female donors. The sex of a patient should be taken into consideration when the concentration of cell-surface-bound NA is determined for diagnostic purposes. As far as cell-surface-bound nucleic acids are concerned, they represent the main part of cirNAs and, along with cirNAs from plasma fraction, provide a valuable source of material for PCR diagnostics (18).

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